Nicotinamide Mononucleotide Adenylyltransferase (NMNAT) Maintains Active Zone Structure by Stabilizing Bruchpilot

Shaoyun Zang, Yousuf O. Ali, Kai Ruan and R. Grace Zhai

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 05 May 2012

Thank you for the submission of your research manuscript to EMBO reports. I am sorry for the delay in getting back to you but we have only now received the full set of referee reports that is copied below. Referee 2 has taken part in a structured referee reports trial and that is why this report comes in a different format.

As you will see, while the referees acknowledge that the study is potentially interesting, they also point out that it requires significant additional experimental confirmation in order to support the conclusions drawn. None of the referees is convinced by the reported specific protection of BRP by NMNAT and all indicate that additional active zone and synaptic vesicle proteins need to be examined in the NMNAT knockdown flies. Along these lines, referee 2 asks for comments on the apparent loss of Synaptotagmin and referee 3 points out that rescue experiments need to be performed to confirm that the observed phenotypes are due to NMNAT knockdown. Referee 3 also mentions that localization of BRP and NMNAT and structural analyses of the active zone need to be investigated in the same type of synapse, that proteasomal degradation of BRP in the absence of NMNAT should be confirmed, that an effect on BRP transcription should be excluded, and that an additional antibody should be used to detect BRP. The referee also pinpoints technical concerns and alternative interpretations of the data that need to be addressed. Referee 2 further mentions that the suggested activity-dependent stabilization of BRP by NMNAT needs to be strengthened, and referee 1 feels that synapse loss should be examined in the NMNAT knockdown brains.
From the referee comments it is clear that the current data are insufficient to support the main conclusion of the manuscript. Publication of the study in our journal can therefore not be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the length of the revised manuscript may not exceed 30,000 characters (including spaces) and maximally 5 figures may be presented in the main manuscript file. Supplementary figures should further directly relate to their corresponding main figures in the manuscript. Please also remember to include statistical analyses for all quantifications performed, and please mention the number of experiments (n=3 or >3), the statistical test used, and the identity of the error bars in the corresponding figure legends (currently missing).

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor

EMBO reports

REFEREE REPORTS:

Referee #1:

The authors here analyze further the role of nicotinamide mononucleotide adenylyltransferase (NMNAT) in its role for the integrity of synapses in Drosophila larval NMJ and adult optical system. The authors previously showed that NMNAT displays chaperone function both in biochemical assays and cultured cells, and that it shares significant structural similarity with known chaperones. Furthermore, it is upregulated in the brain upon over-expression of poly-glutamine expanded protein and recruited with the chaperone Hsp70 into protein aggregates. Here now they further address its role at presynaptic active zones of the fly, forming T bar called dense bodies composed of the large coiled coil domain protein Bruchpilot (BRP). They start by using pan-neuronal RNAi versus NMNAT and report a change in the distribution of BRP in adult fly CNS, with neuropil staining severely being reduced (nearly outspared from BRP!) but somatic aggregates becoming visible. For "control", DLar stainings are done, showing a reduction of neuropil label while somatic aggregates are apparently not forming for this protein. In Fig. 2, BRP and NMNAT levels are determined from head extracts by Western, finding a reduction of BRP in pan-neuronal RNAi versus NMNAT. Next, IPs versus BRP and NMNAT are shown to contain the respective other protein detected by western blot. Next (2C), they IP BRP and probe for ubiquitin protein, reporting an upregulation of Ubiquitin and HSP70 positive immunoreactivity in the BRP IPs after reduction of NMNAT by RNAi. Next, they stain NMJs for NMNAT, reporting a degree of co-localization of between NMNAT and BRP marking active zones. Finally, they study fly photoreceptor dense bodies, measuring the size of the t bar "roofs". They find that photoreceptor neuron -triggered RNAi versus NMNAT reduces T bar size, but only under LD means under "activity" conditions. Thus, they state the maintenance of BRP at the active zone under normal neuronal activity requires the presence of NMNAT. Finally, they expressed HA-tagged NMNAT in the photoreceptors and altered the photoreceptor activity level by rearing under LD or DD conditions. They report a higher amount of BRP in coIP with NMNAT under LD conditions.
The paper touches upon an interesting topic. In its current state, however, the paper fails short on supporting its claims. Most importantly, I am not convinced that the "degeneration" upon removal is really that specifically affecting BRP. For control purposes in their immunostainings, they exclusively use Dlar stainings/blots. First of all, Dlar looks very atypical as well in their brain stainings, and the Dlar antibody might also simply run into its detection limit when using it on blots. It in my eyes would need several protein markers more, including active zone proteins as liprin-a or DRBP, as well as SV proteins (synaptobrevin, synaptotagmin....) to drive the specificity point home. They say "In contrast to BRP, the distribution of another active zone protein DLAR (Stryker & Johnson, 2007) was not altered in NMNAT knock-down brains, although the protein level of DLAR was reduced to a level similar to that of BRP (Figure 1C1-D4, Supplemental Figure 1), likely owing to a loss of synapses." Well, this could be all a matter of sensitivity of their tools. They would need to collect data describing the synaptic situation in their brains, they would need to look into additional proteins etc.

This all said, I do think the study might have potential and would be happy to look into a revised version given the mentioned point could be addressed.

Referee #2:

1. Do the contents of this manuscript report a single key finding? YES / NO

Yes. They identify a synaptic active zone protein BRP as a substrate for NMNAT chaperon activity. They also show that this regulation may play a role in activity-dependent synapse active zone maintenance.

2. Is the main message supported by compelling experimental evidence? YES / NO

Yes. Most biochemical analyses are performed carefully. The morphological effects on active zone are also very striking.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? YES / NO

Although dynamic regulation of synaptic active zones has been reported in various contexts, direct link between NMNAT chaperon and synaptic active zone has not been reported.

4. Is the main finding of general interest to molecular biologists? YES / NO

Yes. The experiments raise interesting questions of how NMNAT chaperon contribute to BRP synaptic localization and abundance.

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

[aX] in EMBO reports
[b] in another of EMBO's titles (if so, which one?)
[c] in a more specialized journal elsewhere
[d] not at all
6. Please add any further comments you consider relevant:

Overall, this is an excellent study revealing an unexpected link between a specific synaptic active zone protein, BRP and NMNAT, a protein that is of central interests to neurogeneration field. The biochemical data are very strong and quantitative. The cellular analyses of synapses and active zones are beautiful and thorough. An interesting finding is the activity-dependent regulation of BRP by NMNAT. However, this conclusion should be further strengthened. It is unclear at which level this activity-dependent regulation acts. For example, does blocking synaptic transmission modify NMNAT synaptic defects and the regulation of BRP by NMNAT?

A minor point: supplemental Fig S1A show loss of Synaptotagmin, which is never commented in the manuscript, nor quantitated. The loss is quite dramatic, comparing to DLAR or BRP. How does the authors interpret this finding?

Referee #3:

Zhang, Ali, et al.

In this manuscript, Zhang, Ali et al describe a role for NMNAT in release site structure. They show that in neurons with reduced NMNAT BRP is excessively ubiquitinated, the protein clusters in inclusions in cell bodies and less BRP immunoreactivity is detected at synapses. At the ultrastructural level, the authors show data that indicate T-bars in fly photoreceptors show morphological defects. Finally, the authors show that BRP co-IPs with NMNAT and that in photoreceptors this interaction increases when the photoreceptors are excessively stimulated by light. The authors propose a model where the chaperone activity of NMNAT is needed to maintain BRP integrity at release sites.

I find this an interesting paper that touches upon a timely topic, that of maintaining "synaptic health" despite intense use and stimulation. While the model is appealing, the data is not always in support and there are also a number of technical concerns that need to be addressed in a revised version:

1) Complete loss of NMNAT is lethal, and therefore, the authors have used RNAi, however, the specificity of the phenotypes was not demonstrated using rescue experiments. Alternatively, why not use the eyFLP system to generate mutant photoreceptor cells using NMNAT alleles that are clean and can be rescued; the immunofluorescence can be done in these photoreceptors and EM as well.

2) The authors show BRP and NMNAT localization in the central fly brain and at motor boutons in larvae, but not in photoreceptor terminals where they perform their ultrastructural analyses. These neuron subtypes are not necessarily similar in their mode of using NMNAT and in the effect of NMNAT on BRP. Why is the immunohistochemistry not shown in photoreceptor terminals in the lamina (a preparation they have used before) and why was the EM of T bars not performed at larval motor neuron endplates?

3) an important aspect of their analyses is that NC82 immunoreactivity is downregulated upon NMNAT loss of function. The model implies increased ubiquitination to be the culprit. First, can this defect be inhibited using proteasomal inhibitors? Second, labeling with several additional synaptic (density) markers (in addition to dLAR) would be appropriate. Third, quantitative RT-PCR will rule out an effect of chronic loss of NMNAT on transcription of BRP.

4) The TEM of photoreceptor contacts in the lamina is of poor quality and previous papers include better images of these terminals. The T-bars appear fuzzy (also in wild types) and other organelles are not very discernible... Furthermore, the quantification of T-bar size is in my view prone to errors. The authors should also see defects in BRP (NC82) morphology using their super resolution SIM imaging... In relation to this, why are the typical 'ring' structures of NC82 labeling (shown by STED and SIM) not visible; the resolution attained by this system should be sufficient.

5) related to the previous point, the defect in NC82 labeling the authors see in NMNAT loss of function is interpreted as a defect to assemble T bars (as is indicated in the model), however, the data is far from solid here. NC82 is a monoclonal antibody, and as was shown in Fouquet et al, it labels the C-end of the protein. If the increased ubiquitination in NMNAT loss of function interferes with antibody binding they would also observe reduced NC82 labeling. The authors can rule out this alternative possibility by labeling NMNAT loss of function with a different BRP antibody as well.
6) An alternative possibility that in my view is also consistent with the data is that NMNAT regulates BRP trafficking from the cell body to the synapse. BRP is seen to accumulate in inclusions away from the synapse upon loss of NMNAT and less BRP detected by NC82 appears to be present at synapses (however, see pt. 5), NMNAT and BRP bind, upon activity more NMNAT binds to BRP (exactly when more BRP needs to be delivered to synaptic contacts) and in wild types, BRP and NMNAT are present in close proximity at synapses, where NMNAT would deliver the BRP protein. Can the authors rule out this possibility or discuss it?

7) the potential regulation of dynamic changes in active zone structure during neuronal activity as stated in the abstract and implied in the text was not shown in this work.

8) In fig 2C, unless I am misinterpreting the data, I would have expected to see a polyubiquitin signal in the input of controls as well. Why is no ubiquitin signal detected? Furthermore, in 2C, the data implies multiple ubiquitin chain lengths on BRP, yet in F, only one major ubiquitinated BRP variant appears present; would one not expect a 'smear' as well here?

9) the text is in general well written, but there are a number of grammatical and spelling mistakes that should be taken care of.

1st Revision - authors' response

Response to reviewers’ comments.

Referee #1:

The authors here analyze further the role of nicotinamide mononucleotide adenylyltransferase (NMNAT) in its role for the integrity of synapses in Drosophila larval NMJ and adult optical system. The authors previously showed that NMNAT displays chaperone function both in biochemical assays and cultured cells, and that it shares significant structural similarity with known chaperones. Furthermore, it is upregulated in the brain upon over-expression of poly-glutamine expanded protein and recruited with the chaperone Hsp70 into protein aggregates.

Here now they further address its role at presynaptic active zones of the fly, forming T bar called dense bodies composed of the large coiled coil domain protein Bruchpilot (BRP). They start by using pan-neuronal RNAi versus NMNAT and report a change in the distribution of BRP in adult fly CNS, with neuropil staining severely being reduced (nearly outspared from BRP!) but somatic aggregates becoming visible. For "control", DLar stainings are done, showing a reduction of neuropil label while somatic aggregates are apparently not forming for this protein. In Fig. 2, BRP and NMNAT levels are determined from head extracts by Western, finding a reduction of BRP in pan-neuronal RNAi versus NMNAT. Next, IPs versus BRP and NMNAT are shown to contain the respective other protein detected by western blot. Next (2C), they IP BRP and probe for ubiquitinated protein, reporting an upregulation of Ubiquitin and HSP70 positive immunoreactivity in the BRP IPs after reduction of NMNAT by RNAi.

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This all said, I do think the study might have potential and would be happy to look into a revised version given the mentioned point could be addressed.

>>We thank the reviewer for recognizing the potential of our work and the encouraging comments. We have followed the reviewer’s suggestions and carried out additional experiments to determine the specificity of the effects of nmnat knockdown.

First, we examined the expression of DLAR with western analysis and immunostaining. The DLAR antibody works when used in high concentration (1:500 for immunostaining and 1:200 for Western blotting). We clearly observe the DLAR-specific band at around 240 kDa (supplementary Fig. S1A) and also observe the previously reported DLAR staining in synaptic neuropil structures in the adult midbrain (supplementary Fig.S2A-2B).

Second, we examined additional synaptic markers as suggested by Reviewer 1, including synaptotagmin and CSP proteins (Supplementary Fig. S2). As previously reported, immunostaining of both synaptotagmin and CSP is enriched in adult brain neuropil structures. Knockdown of NMNAT also reduced the protein level of synaptotagmin and CSP, which is consistent with DLAR reduction in NMNAT-RNAi neurons (supplementary Fig. S1). Indeed, as the reviewer pointed out, the observed reduction in several synaptic protein levels suggests that a general loss of synapses resulted from pan-neuronal knockdown of NMNAT. This is consistent with previous findings in mammalian systems where NMNAT1, for example, is known to be a potent neuroprotective agent at the synapse and in axons [1] However, in contrast to BRP, whose distribution was altered in NMNAT-knockdown neurons, the localization of DLAR, synaptotagmin and CSP was not affected by NMNAT knockdown, further suggesting that the distribution of BRP was specifically affected by loss of NMNAT.

Third, we investigated the ubiquitination of synaptic proteins in NMNAT-knockdown neurons. In contrast to BRP, the ubiquitination of which was increased in NMNAT-knockdown neurons, ubiquitinated DLAR, synaptotagmin or CSP was not observed (Supplementary Fig. S4), further suggesting that the ubiquitination and degradation of BRP was regulated specifically by NMNAT.

In summary, these analyses on several synaptic proteins in addition to BRP suggest that first, NMNAT is required to maintain synaptic integrity where knockdown of NMNAT in the CNS induces a loss of synapses and synaptic proteins; and second, NMNAT plays a specific role in maintaining BRP protein stability and its localization at the synapse. We have incorporated these results in the revised version, in Supplementary Figs. S1, S2, S4, and in the results section on pages 5-6.

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>>We thank the reviewer for the encouraging comments on our findings. We have followed the reviewer’s suggestion and carried out additional experiments to strengthen our activity-dependent observations. Our previous studies have shown that nmnat null photoreceptors undergo rapid activity (light)-induced degeneration, resulted from a lack of NMNAT-mediated neuronal maintenance [2]. In this report, we show that NMNAT maintains the localization and level of BRP in an activity-dependent manner as well (Fig. 4). To further strengthen this point, we carried out an additional activity blocking experiment as suggested by the reviewer. We took advantage of a mutation in NorpA (no receptor potential A [3]) where the phototransduction cascade and synaptic transmission in photoreceptors are blocked/attenuated. We introduced the NorpA mutation in the background of nmnat null and analyzed the synaptic structures in NorpA and nmnat double mutant photoreceptor terminals. By ultrastructural analysis, we found that in nmnat null terminals the active zone (T-bar) size declined with age under normal (dark/light) conditions, indicating age- and activity-dependent synaptic degeneration. However, we observed larger T-bars in NorpA/nmnat double mutant photoreceptor terminals, compared to those in nmnat null terminals at the same age (Fig. 4F,4H), indicating reduced degeneration. These results, together with the observation that increased activity leads to increased NMNAT-BRP interaction (Fig. 4J-I), suggest that under normal activity conditions, active zones/T-bars are dynamic structures that require maintenance by NMNAT. When neuronal activity is attenuated, either by blocking light stimulation (rearing in the dark), or by blocking phototransduction (NorpA), the requirement for NMNAT-mediated maintenance is reduced. We have incorporated these results in the revised version, in Figs. 4E, 4F, and 4H, and in the results section on pages 8-9.

A minor point: supplemental Fig S1A show loss of Synaptotagmin, which is never commented in the manuscript, nor quantitated. The loss is quite dramatic, comparing to DLAR or BRP. How does the authors interpret this finding?

>>We thank the reviewer for pointing this out. We carried out additional experiments to further analyze the effect of NMNAT knockdown on additional synaptic proteins including synaptotagmin. We observed by immunostaining (Supplementary Fig. S2) and by western
blot analysis (Supplementary Fig. S1) that the synaptotagmin level is largely reduced in NMNAT knockdown neurons. In addition, other synaptic proteins, including DLAR and CSP, are also reduced, suggesting a loss of synapses upon knockdown of NMNAT.

Although all synaptic proteins that we have examined showed reduced levels in NMNAT knockdown brains, the loss of synaptotagmin is most severe (15% of control) (Supplementary Fig. S1). This could be due to the difference in degradation pathways and clearance dynamics for synaptic vesicle-associated proteins vs. cytomatrix- and plasma membrane-associated proteins. We have included the analysis of protein levels and incorporated these results in the revised version, in Supplementary Figs. S1 and S2, and in the results section on page 5.

Referee #3:

Zhang, Ali, et al.

In this manuscript, Zhang, Ali et al describe a role for NMNAT in release site structure. They show that in neurons with reduced NMNAT BRP is excessively ubiquitinated, the protein clusters in inclusions in cell bodies and less BRP immunoreactivity is detected at synapses. At the ultrastructural level, the authors show data that indicate T-bars in fly photoreceptors show morphological defects. Finally, the authors show that BRP co-IPs with NMNAT and that in photoreceptors this interaction increases when the photoreceptors are excessively stimulated by light. The authors propose a model where the chaperone activity of NMNAT is needed to maintain BRP integrity at release sites.

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1) Complete loss of NMNAT is lethal, and therefore, the authors have used RNAi, however, the specificity of the phenotypes was not demonstrated using rescue experiments. Alternatively, why not use the eyFLP system to generate mutant photoreceptor cells using NMNAT alleles that are clean and can be rescued; the immunofluorescence can be done in these photoreceptors and EM as well.

>> We thank the reviewer for pointing this out. We followed the reviewer’s suggestions and carried out the following lines of experiments.

First, we used MARCM (with eyFLP) analysis to examine BRP and NMNAT staining in nmnat null photoreceptor terminals, and observed reduced BRP levels in nmnat null terminals, compared to the neighboring nmnat heterozygous terminals (Fig 1B), consistent with our findings using the RNAi-mediated knockdown approach (Fig 1C-1D’).

Second, to confirm the specificity of NMNAT RNAi-mediated knockdown, we carried out a rescue experiment using a transgenic line UAS-human NMNAT3, of which the transcript is not recognized by the Drosophila NMNAT RNAi. We observed that when NMNAT was knocked down in motor neurons, flies failed to eclose and died at the pupal stage, a phenotype that can be rescued completely by overexpression of human NMNAT3 (Supplementary Fig S3). The rescue of eclosion rate by human NMNAT3 expression suggests that Drosophila NMNAT RNAi-mediated knockdown specifically caused a loss of function of nmnat.

As eyFLP-induced loss of function is restricted to the photoreceptors, and therefore precluded the feasibility of biochemical analysis, we employed an RNAi-mediated knockdown method that can be applied to the entire brain to carry out all the biochemical analyses. In this report, we used both eyFLP generated nmnat null neurons and RNAi-mediated NMNAT knockdown neurons to carry out the analysis in order to take advantage of both approaches.
We included the results of the new experiments in Fig. 1B1-B4, Supplementary Fig. S3, and incorporated the information in the revised version, on pages 4-5.

2) The authors show BRP and NMNAT localization in the central fly brain and at motor boutons in larvae, but not in photoreceptor terminals where they perform their ultrastructural analyses. These neuron subtypes are not necessarily similar in their mode of using NMNAT and in the effect of NMNAT on BRP. Why is the immunohistochemistry not shown in photoreceptor terminals in the lamina (a preparation they have used before) and why was the EM of T bars not performed at larval motor neuron endplates?

>> To address the points raised by the reviewer here, we carried out an additional MARCM experiment (with eyFLP) to further analyze the effect of loss of NMNAT in photoreceptor synapses. We observed that BRP staining colocalized with NMNAT staining in the lamina, and observed reduced BRP levels in nmnat null terminals, compared to the neighboring nmnat heterozygous terminals (Fig 1B1-B4). This is consistent with our findings in brain using the RNAi-mediated knockdown approach (Fig 1C1-D4’). To further analyze the localization of NMNAT and BRP, we used super resolution fluorescence microscopy. Drosophila larval NMJ is suitable for super resolution (250 nm in XY) microscopy because of its large size and relatively flat structure. Previous studies on the active zone localization of BRP have also used super resolution microscopy and elegantly demonstrated the precise active zone anchoring of BRP in the larval NMJ [4, 5]. By means of super resolution microscopy, we observed the typical ‘ring’ structures of NC82 labeling and the colocalization between NMNAT and BRP ‘ring’ structures, suggesting possible direct interaction between BRP and NMNAT (also see point # 4 below). These results are included in Figure 3C1-C3. Indeed the regulatory mechanisms in different neuronal subtypes might differ; however, it has been shown that BRP plays a similar structural role in different synapses [4, 5] and that NMNAT has been found to be a maintenance/protective factor in multiple neurons [2, 6, 7]. Therefore we would like to investigate whether the interaction between NMNAT and BRP is cell type-dependent and examine this interaction in different synapses. We now show that NMNAT interacts with BRP in photoreceptor terminals, in central brain, and in larval NMJ (with super resolution). These results suggest that NMNAT and BRP interact in different neurons and synapses. We have included this information in Figs. 1 and 3 and incorporated these results on page 7 in the revised version.

3) an important aspect of their analyses is that NC82 immunoreactivity is downregulated upon NMNAT loss of function. The model implies increased ubiquitination to be the culprit. First, can this defect be inhibited using proteasomal inhibitors? Second, labeling with several additional synaptic (density) markers (in addition to dLAR) would be appropriate. Third, quantitative RT-PCR will rule out an effect of chronic loss of NMNAT on transcription of BRP.

>> We thank the reviewer for these excellent suggestions. We carried out the following lines of experiments to address each of these points.

First, we introduced a proteasomal inhibitor, MG132, by feeding fly larvae for 24 hours [8] and examined the level of BRP ubiquitination in wild-type or NMNAT overexpression flies. To specifically analyze the ubiquitinated pool of BRP protein, we immunoprecipitated BRP, probed with both anti-BRP and anti-ubiquitin antibody, and determined the percent of ubiquitinated BRP using multiplex western analysis, where only the protein bands detected by both BRP and ubiquitin antibodies were considered as ubiquitinated BRP (Supplementary Fig. S5A, arrows indicate the ubiquitinated BRP bands, shown in yellow in the merged image). In wild-type flies, the percentage of ubiquitinated BRP increased when proteasome function was inhibited with MG132 (Fig. 2E, Supplementary Fig. S5B), indicating an accumulation of ubiquitinated BRP upon inhibited proteasome function. In NMNAT-overexpressing flies with 3 copies of nmnat genes, the percent of ubiquitinated BRP was lower than that in wild-type with either DMSO or MG132 treatment (Fig. 2E, Supplementary Fig. S5), suggesting that the ubiquitin proteasome pathway is involved in regulating BRP protein degradation and that a higher level of NMNAT reduces the ubiquitination of BRP. We also tried to treat NMNAT RNAi-flies with MG132; however, the treatment resulted in early death within 24 hours of drug treatment and precluded
biochemical analysis. These results suggest that the BRP protein level is regulated by ubiquitination and the proteasomal pathway and that the level of NMNAT is a critical regulator of this process. We included this information in Fig. 2E and supplemental Fig. S5, and incorporated these results in the revised version, on pages 6-7.

Second, we examined the effect of NMNAT knockdown on other synaptic markers, including DLAR, synaptotagmin, and CSP and included the results in Supplementary Fig. S1 and S2. As previously reported, immunostaining of both synaptotagmin and CSP is enriched in adult brain neuropil structures. Knockdown of NMNAT also reduced the protein level of synaptotagmin and CSP, which is consistent with DLAR reduction in NMNAT RNAi-neurons (supplementary Fig. S1). The reduction of several synaptic proteins suggests that a general synapse loss resulted from pan-neuronal knockdown of NMNAT. This is consistent with previous findings in mammalian systems where NMNAT1, for example, is known to be a potent neuroprotective agent at the synapse and in axons [1]. However, in contrast to BRP, whose distribution was altered in NMNAT knockdown neurons, the localization of synaptotagmin and CSP was not affected by NMNAT knockdown, further suggesting that the distribution of BRP was specifically affected by loss of NMNAT.

Third, we carried out real-time PCR analysis to determine the transcription level of BRP. We observed that BRP transcripts were slightly increased upon NMNAT knockdown (Fig. 2D). Therefore, the reduction in BRP protein level is not due to reduced transcription.

4) The TEM of photoreceptor contacts in the lamina is of poor quality and previous papers include better images of these terminals. The T-bars appear fuzzy (also in wild types) and other organelles are not very discernible... Furthermore, the quantification of T-bar size is in my view prone to errors. The authors should also see defects in BRP (NC82) morphology using their super resolution SIM imaging... In relation to this, why are the typical ‘ring’ structures of NC82 labeling (shown by STED and SIM) not visible; the resolution attained by this system should be sufficient.

>> In this report, we employed fluorescence confocal microscopy, super resolution microscopy, EM, and biochemical analysis to take advantage of the strength of each technique and address one question. EM offers ultrastructural analysis but a limited view of individual terminals/synapses in an animal. As the reviewer rightfully pointed out, EM quantification has a low throughput. However, EM is the most direct way to visualize the morphology of the active zone, a protein-dense structure of ~200 nm in size. To overcome potential errors in EM quantification, we increased sample size (N) and employed double-blind quantification, where the quantification was done while blinded to the genotypes. With additional EM analysis, we found that loss of nmnat induced T-bar degeneration (measured by T-bar platform width) in an age-dependent manner (Fig. 4G), and that blocking neuronal activity by NorpA reduced the level of degeneration (Figs. 4E, 4F, and 4H). We incorporated these results in the revised version, on pages 8-9.

We carried out super resolution fluorescence microscopy to support and supplement the findings from EM analysis, and observed the typical ‘ring’ structure of NC82 labeling by Z-stack visualization and the colocalization between NMNAT and BRP ‘ring’ structures, supporting a possible direct interaction between BRP and NMNAT (Fig. 3C1-C3). We incorporated these results in the revised version, on page 7.

5) related to the previous point, the defect in NC82 labeling the authors see in NMNAT loss of function is interpreted as a defect to assemble T bars (as is indicated in the model), however, the data is far from solid here. NC82 is a monoclonal antibody, and as was shown in Fouquet et al, it labels the C-end of the protein. If the increased ubiquitination in NMNAT loss of function interferes with antibody binding they would also observe reduced NC82 labeling. The authors can rule out this alternative possibility by labeling NMNAT loss of function with a different BRP antibody as well.

>> We agree with the reviewer that a different BRP antibody would rule out the alternative possibility that ubiquitination of BRP might interfere with NC82 antibody binding. Unfortunately, NC82 is the only commercially available antibody and we were unable to obtain other antibodies to do the suggested experiment. However, we were able to detect...
ubiquitinated BRP with NC82 antibody (supplementary Fig.5), and when flies were treated with MG-132, the ratio of ubiquitinated BRP (the band which was recognized by both ubiquitin antibody and NC82 antibody) to total BRP increased, suggesting that the NC82 antibody is able to detect both ubiquitinated and native forms of BRP.

6) An alternative possibility that in my view is also consistent with the data is that NMNAT regulates BRP trafficking from the cell body to the synapse. BRP is seen to accumulate in inclusions away from the synapse upon loss of NMNAT and less BRP detected by NC82 appears to be present at synapses (however, see pt. 5), NMNAT and BRP bind, upon activity more NMNAT binds to BRP (exactly when more BRP needs to be delivered to synaptic contacts) and in wild types, BRP and NMNAT are present in close proximity at synapses, where NMNAT would deliver the BRP protein. Can the authors rule out this possibility or discuss it?

>> The reviewer has raised an interesting and important point. The observation of clustered BRP protein in the cell body away from the synapse during loss of NMNAT neurons indicates a possible role of NMNAT in facilitating the transport of BRP. Our work provided the following pieces of evidence supporting the notion that NMNAT is not required for the anterograde transport of BRP and its localization during active zone assembly. First, we showed that in nmnat-null neurons, the active zone structure was formed properly, and was maintained when the light stimulation was blocked (Fig. 4), suggesting that T-bar assembly and maintenance during the absence of activity do not require NMNAT protein. Therefore during synaptogenesis, BRP can be delivered to synaptic terminals without NMNAT. Second, under normal conditions, most of the BRP protein is present at the active zone, and NMNAT protein is localized to the active zone area to carry out the maintenance function and regulate BRP protein turnover locally at the site. Although NMNAT is not required during active zone assembly, we cannot rule out the possibility that NMNAT might be required for anterograde transport of BRP in active zone turnover. We have further discussed this point in the revised version, on pages 10-11.

7) the potential regulation of dynamic changes in active zone structure during neuronal activity as stated in the abstract and implied in the text was not shown in this work.

>> In accordance with this point, we have revised the abstract and introduction to elucidate our findings more accurately and precisely.

8) In fig 2C, unless I am misinterpreting the data, I would have expected to see a polyubiquitin signal in the input of controls as well. Why is no ubiquitin signal detected? Furthermore, in 2C, the data implies multiple ubiquitin chain lengths on BRP, yet in F, only one major ubiquitinated BRP variant appears present; would one not expect a ‘smear’ as well here?

>> In wild-type flies under normal conditions, the level of protein ubiquitination is relatively low. We typically load 1/10 in the input lane and at such a concentration, ubiquitination signals were below the detection limit. Therefore no polyubiquitin signal was seen in control inputs in Fig. 2. To further analyze the ubiquitination of BRP, we carried out additional experiments. To enhance ubiquitination, we introduced a proteasomal inhibitor, MG132, as suggested by the reviewer (see point #3 above). In wild-type flies, the level of ubiquitinated BRP increased with MG132 treatment, suggesting an accumulation of ubiquitinated protein with inhibited proteasome function. We observed a major ubiquitinated BRP variant in the background of a ‘smear’ detected by anti-ubiquitin antibody (Fig 2E), indicating a major poly-ubiquitin modification among multiple ubiquitin chain lengths. Using anti-BRP antibody we detected a major up-shifted band, suggestive of ubiquitinated BRP. To specifically analyze the ubiquitinated pool of BRP protein, we carried out multiplex western analysis. We immunoprecipitated BRP protein from brain lysates, and probed with anti-BRP and anti-ubiquitin antibodies simultaneously. The protein bands detected by both BRP and ubiquitin antibodies were considered as ubiquitinated BRP, and the percent of ubiquitinated BRP over total BRP was determined (Supplementary Fig. S5A, arrows indicate ubiquitinated BRP bands, colored yellow in the merged image). In NMNAT overexpression flies (three copies of the nmnat genomic region), the increase of ubiquitinated BRP level with MG132 treatment was diminished (supplemental Fig S5), although the modification pattern is similar. We were unable to
obtain MG132-treated NMNAT RNAi-knockdown flies as they died with 24 hours of drug treatment. A lack of 'smear' background in BRP blots is likely because of a dominant ubiquitinated BRP variant recognized by the BRP antibody. We included these additional results in Fig. 2E, supplemental Fig. S5, and incorporated the information and discussion in the revised version, on pages 6-7.

9) the text is in general well written, but there are a number of grammatical and spelling mistakes that should be taken care of.

>>We have carefully revised the text to correct all grammatical and spelling mistakes.

References

2nd Editorial Decision 11 October 2012

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. Both referees still have a few minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript. I think all of the comments raised by referee 3 should be addressed, point 1 by adding the quantifications and the higher magnification images of the T-bars and point 2 at least in the manuscript text. While the quantifications referee 1 is asking for would certainly be a useful addition and strengthen the data (and may be easy to do), we also feel that they are not absolutely required.

I have also noticed that the manuscript does not contain a materials and methods section. Basic materials and methods essential to the understanding of the experiments must be described in the main body of the manuscript and may not be presented as supplementary information. Please include at least a brief description of the most important materials and methods in the main manuscript file. You can still add an additional 1000 characters to the text, but beyond that other parts of the text need to be shortened.
I am looking forward to receiving a new, revised version of the manuscript as soon as possible.

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

Many concerns have been addressed in the current version. Still, the manuscript is not fully convincing at places, particularly concerning the comparison between BRP label and the label for other synaptic proteins in brains after NMNAT RNAi. My major concern was about the specificity of the phenotype. I am still not fully convinced that loss of NMNAT affects BRP clustering that specifically but not other synaptic proteins as well. Still other markers would be needed, and importantly brain stainings would have to be quantified. The sheer quality of the stainings is not really satisfying.

Concerning NMNAT label in super-resolution: this would need a NMNAT-/ control to prove specificity of the label.

Referee #3:

The paper has fantastically improved and will make a nice contribution to the field. I only have a couple of minor issues (below) that I hope can be addressed in the final version of the paper. I have also looked over the comments of reviewer 2 and in my opinion also his/her issues have all be addressed (see below).

1) The SIM data in Fig 3B and C are interesting, but I would like to suggest two additions: a) quantification of the % colocalization and b) show a higher magnification inset of one or a few T bars; the current view is a bit too low magnification to be able to carefully assess localization.

2) I had raised the possibility that NMNAT is involved in BRP transport from cell bodies to synapses, explaining the accumulation of T bar material in the cell body and less NC82 labeling at synapses. The authors argue against this possibility based on the following statement "First, we showed that in nmnat-null neurons, the active zone structure was formed properly, and was maintained when the light stimulation was blocked (Fig. 4), suggesting that T-bar assembly and maintenance during the absence of activity do not require NMNAT protein. Therefore during synaptogenesis, BRP can be delivered to synaptic terminals without NMNAT." However, this argument only holds true if also under blocked light stimulation conditions, BRP is accumulated in the nucleus. I don't think this 'control' experiment was shown, and may/should be included. The discussion on this that states "The interesting observation of clustered BRP protein in the cell body away from the synapse in loss-of-NMNAT neurons indicates a possible role for NMNAT in facilitating the transport of BRP" on pg 10 also seems a bit contradictory to the statement on pg 11 "Our results also indicate that NMNAT <specifically> regulates the ubiquitination and degradation of BRP at the synapse"; maybe best to rephrase this...

3) Reviewer 2's main issue was to test the effect of NMNAT on BRP under conditions where synaptic activity was blocked. The authors have used a phototransduction mutant norpA that blocks the generation of the graded potential in the fly eye to test the relation between neuronal activity and NMNAT action towards BRP. They report that blocking activity 'protects' the T-bars, also in nmnat mutants. Based on these data the authors conclude that under normal conditions Tbars are dynamic structures that require maintenance by NMNAT. I believe the new data address the issue raised by the reviewer.

The minor comment by reviewer 2 overlaps with a comment from reviewer 1 and was also addressed.
We would like to thank you and the reviewers again for excellent comments and suggestions. We have followed your recommendations and incorporated the following changes in this new version.
1. We added the quantifications of colocalization between BRP and NMNAT in 3D-SIM™ Super-Resolution imaging (Figure 3D) and the higher magnification images of the T-bars from 3D-SIM™ (Figure 3C').
2. We addressed the point about BRP transport and rewrote the relevant section on page 10.
3. We added a summary of materials and methods in the main body of the manuscript.
4. We have shortened the manuscript text and now the total character count (including spaces) of all sections is 29,938.

The new version of the manuscript is clearer and more concise. We trust that you will find the revised manuscript acceptable for publication.

I am very pleased to accept your manuscript for publication in the next available issue of EMBO Reports. Thank you for your contribution to our journal.

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